APPENDIX A

1. (Amended) An isolated non-murine mammalian pluripotential cell which can:

   (a) be maintained on feeder layers for at least 20 passages; and

   (b) give rise to embryoid bodies and multiple differentiated cell phenotypes in monolayer culture, wherein said cell has the potency characteristics of a cell derived from a primordial germ cell by the process of: (1) culturing a non-murine mammalian primordial germ cell in a composition comprising basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor; (2) selecting cells that have characteristics (a) and (b) above, and (3) isolating said non-murine pluripotential cell; and wherein the characteristics of the cell include having a normal karyotype.

2. The embryonic stem cell of claim 1, having a mutation which renders a gene non-functional.

3. The embryonic stem cell of claim 1, having an insertion of a functional gene.

4. (Amended) An isolated human pluripotential cell which can:

   (a) be maintained on feeder layers for at least 20 passages; and

(b) give rise to embryoid bodies and multiple differentiated cell phenotypes in monolayer culture, wherein said cell has [all] the [essential] potency characteristics of a cell derived from a primordial germ cell by the process of: (1) culturing a human primordial germ cell in a composition comprising basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, and soluble steel factor; (2) selecting cells that have characteristics (a) and (b) above, and (3) isolating said human pluripotential cell; and wherein the characteristics of the cell include having a normal karyotype.

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Embryonic Stem Cells

PART ONE: HISTORY AND USE

By Mark Wight

As a leading supplier of sera, media, and other cell culture reagents, HyClone strives to keep abreast of innovations and advances in cell culture and to obtain a degree of knowledge and expertise in the major areas of research utilizing cell culture. One such area is the culture of embryonic stem cells (ES cells). The number of studies using ES cells has increased considerably over the past decade with a corresponding increase in the demand for specialty sera and media specific for this application. What are ES cells? When were they discovered? What is being done with them? This article addresses these and other questions. The next articles in this series will discuss the culture and manipulation of these cells.

ES cells are cells derived from a pre-implantation embryo. These cells are pluripotent, meaning that under different stimuli they can develop and become different types of tissue. A single ES cell could, under certain conditions, divide into the cells that form cardiac muscle. Under different conditions, that same cell could develop into the cells that form gametes. This ability to differentiate into all types of tissue is retained from the embryo from whence the cells originated and is what makes these cells valuable as a research tool. Appropriate culture conditions play an integral part in retaining this pluripotent ability of the cells, thus explaining the necessity for specialty sera and media.

By 1980, pluripotent cells derived from mouse teratocarcinomas had been cultured successfully. Teratocarcinomas are rare and unique tumors that develop from an embryo and contain a wide variety of differentiated tissue (hair, teeth, toes) as well as pockets of undifferentiated, pluripotent cells (1). The cell lines established from these pockets are called embryonal carcinoma (EC) cells and are similar in many ways to undifferentiated cells of a normal embryo. These EC cells were somewhat of a precursor to the ES cells that are so widely used today. While these cells were useful as a tool for studying the development and differentiation of the early embryo, they had limitations: isolation of these cells was complicated and involved, the user was restricted as to which strain of mouse could be utilized, the cells had an abnormal chromosome complement and most importantly, the EC cells were inefficient at contributing to the formation of chimeras (2, 3). It was theorized that pluripotent cells isolated directly from the embryo would overcome some of these limitations and thus have advantages over EC cells (4).

In 1981 two articles, one by Evans and Kaufman (4), and one by Martin (5), reported the isolation of pluripotent cell lines directly from the mouse embryo. Evans and Kaufman termed the cells they isolated "EK"cells indicating that these cells carried a normal karyotype. They used "delayed blastocysts" as the source material for their EK cells. These embryos were prevented from implanting and developing past the blastocyst stage by removing the

continued p. 2

VOL. 17 NO. 3

1 9 9 8

IN THIS ISSUE

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maternal hormones with an ovariectomy followed by administration of progesterone (6). This procedure allowed the cell number to increase while preventing further differentiation (4). They used STO (a mouse embryonic fibroblast line) feeder layers to isolate these EK cells (the STO feeders were commonly used in the culture of EC cells). Martin named her cells embryonic stem (ES) cells. These cells were isolated from normal pre-implantation embryos (blastocysts) that had undergone immunosurgery. Immunosurgery involves treating the embryo with rabbit anti-mouse serum and guinea pig complement. This destroys only the outer layers of cells, leaving the inner cell mass (ICM) where the pluripotent progenitors of ES cells are found (7). They used media conditioned by an established EC cell line for isolation of these ES cells. These two reports were the first to report cell lines of what are now known as embryonic stem cells.

Soon after their introduction, ES cells were shown to be capable of contributing to the development of a wide variety of tissues in chimeric mice, including the germline (8). Embryos were injected with the ES cells and then returned to a foster mother, where the embryos developed normally into mice with tissues inherited from the ES cells. Some of these chimeras were able to pass the ES inherited traits on to the next generation and thus demonstrated the ability to produce germ-line chimeras using ES cells.

In 1985 research on homologous recombination (HR) culminated with the report of successful HR in cultured mammalian cells (9). HR allows for the insertion of genes into the genome at a specific site or the disruption of specific genes already present in the genome. Using HR to add or destroy specific

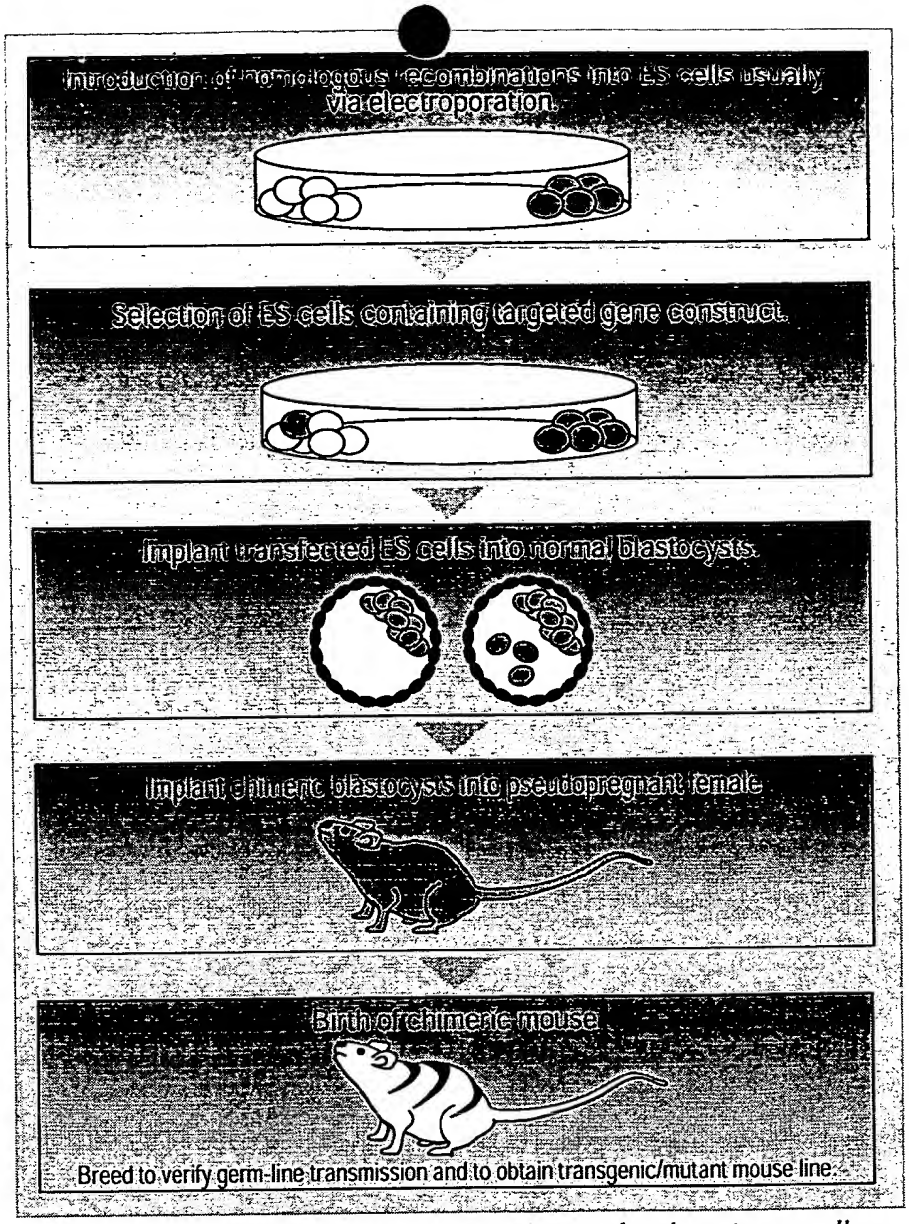

Figure 1. Steps involved in producing chimeric mice with embryonic stem cells.

genes is known as gene targeting. Gene targeting, combined with the ability of ES cells to form germline chimeras, has revolutionized the study of mammalian genetics.

Using ES cells for the production of germline chimeras harboring a created mutation was reported in 1986 (10, 11). In one instance, a retrovirus was integrated into ES cells used to produce transgenic mice (11) and in the other, calcium phosphate mediated transfection was used to introduce the neo^r gene (10). Hooper et al. (12), and Kuehn et al. (13) demonstrated the first instance of modifying a specific gene in 1987. Both groups produced mutants of the hypoxanthine phosphoribosyl transferase (HPRT) gene. Hooper selected preexisting mutants, while Kuehn disrupted the gene with a retrovirus. They both were successful in producing germline mice deficient in HPRT. The hope was that the transgenic mice could be used as a model for Lesch-Nyhan disease. Unfortunately, the mice did not show the symptoms of the disease due to metabolism differences between mice and humans (14). Regardless, both Hooper and Kuehn showed that it is possible to introduce specific genetic changes into the germline of a mouse model.

The first instances of using homologous recombination to target a specific location for genetic manipulation in ES cells were also accomplished with the HPRT gene (15, 16). The first germline transmissions of mutations in genes other than HPRT were accomplished in 1989. In one instance this was done using the c-*abl* gene (17) and in the other the $ß_2$-microglobulin gene was used (18). In 1990 ES cells were shown capable of producing germline chimeras after culture in the absence of feeder layers (19, 20).

Since their discovery, ES cells have become powerful tools in the hands of researchers. Taking advantage of the pluripotent capabilities of these cells, investigators are able to produce mice harboring a mutation in any known gene. ES cells can also be manipulated *in vitro* to develop into various types of differentiated tissue, thus providing *in vitro* models to aid in the study of differentiation.

To create transgenic mice containing a desired genetic change, researchers use gene targeting to manipulate a specific gene in the ES cell genome and then allow the cells to take part in the development of mice. The procedure involves first culturing the ES cells under conditions which will retain the pluripotent characteristics of the cells. Next, the ES cells are introduced to a targeting vector, usually via electroporation, allowing transfection by the vector. Those cells that have successfully integrated the vector at the desired location are then identified using various selection systems. The selected cells are then injected directly into a normal blastocyst embryo, which is then transferred to a pseudopregnant female where the cells participate in the development and eventual birth of a chimeric mouse (see figure 1).

This technique is being used to create mouse models of human disease and promises to take advantage of information provided by the human genome project. ES cells have the potential to provide mouse models to aid in the understanding of, and in the development of therapies for, the more than 5,000 different genetic diseases in humans (21, 22). As the mouse genome is estimated to differ by less than 1% from the human genome (22), information provided by these mouse models will likely be applicable to the corresponding disease in humans. Cancer researchers have used this technology to better understand the role of tumor suppressor genes (22) and oncogenes (21). Immunologists are using gene targeting to unravel the roles of genes involved with the production and function of T and B lymphocytes (22). Gene targeting has aided in the understanding of cystic fibrosis(21) and Di George syndrome (22). Rather than list each area of research that has benefitted by the use of ES cells (as there are many areas of study profiting from this technology) it suffices to say that these cells have contributed to our knowledge of genetic disorders and are contributing toward better therapies. Note that the use of this powerful technology extends to the analysis of the action of any known gene, whether or not the gene is involved in any disorder.

ES cells can also be directed *in vitro* to differentiate into visceral and parietal endoderm , smooth muscle, cardiac muscle, hematopoietic cells, endothelial cells, nerve cells, melanocytes and other cell types (23, 24, 25, 26). These cells can then provide *in vitro* models for various studies. Use of ES cells for *in vitro* cell differentiation eliminates some of the complications involved in an animal model and provides the researcher a more controlled, rapidly developing system (25). In certain cases, an *in vivo* model is not possible as the knockout of the targeted gene causes poor chimera formation or embryo death (23), and an *in vitro* model may be the only viable option.

Also of interest is that ES or similar cells have been isolated from species other than mouse. Species from which ES or ES-like cells have been obtained include pigs (27), rabbits (28), hamsters (29), cattle (30), mink (31), rats (32), sheep (27), chicken (33), quail (33), primates (34), and humans (35). To date, most of these cells have shown limited value, but as investigations progress, obtaining cells with capabilities similar to the mouse ES cells will most likely become a reality. One interesting and recently reported method involves using nuclear transfer techniques to produce ES like cells which are able to form chimeras in cattle (30).

In summary, ES cells are pluripotent cells originating from the ICM of a blastocyst stage embryo. These cells were originally isolated in the early 1980s and have become influential in the study of gene function. They are used to create mouse models for these studies and are also used for *in vitro* differentiation studies. ES cells have also been isolated from species other than the mouse. For further review, references are available (2, 3, 14, 36, 37). Part two of this article will delve into the specifics of the culture conditions required for, and manipulations performed on, these cells. HyClone's interest in ES cells stems from the desire to keep informed and educated about advances in cell culture technology and to meet the need for specialty products. HyClone continues to offer Fetal Bovine Serum that has been screened for the ability to promote the rapid growth of ES cells while retaining their pluripotent abilities. Please contact your HyClone sales representative for more information. □

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Reviews of chromosome studies in urological tumors. III. Cytogenetics and genes in testicular tumors.

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PURPOSE: We reviewed available cytogenetic and molecular findings in testicular germ cell tumors, and their possible application to clinical, pathological and basic parameters. MATERIALS AND METHODS: Findings in the literature on testicular germ cell tumors as well as those from our laboratory were summarized, including a listing of the cytogenetic findings on testicular germ cell tumors to date with some illustrations. RESULTS: Testicular germ cell tumors are characterized in most cases by the presence of an i(12p) isochromosome. In tumors without such an abnormal chromosome studies using fluorescence in situ hybridization and molecular approaches have demonstrated either masking of the i(12p) or the presence of extra 12p sequences in the karyotype. Although testicular germ cell tumors are often associated with chromosome changes in addition to the i(12p), no other specifically recurrent structural chromosome changes have been found. Based on the cytogenetic and molecular findings in testicular germ cell tumors, a hypothetical scheme for the genetic events leading to these tumors is presented. CONCLUSIONS: The genetic events leading to genesis of testicular germ cell tumors in men appear to be related to aneuploidization followed by the formation of an i(12p) isochromosome, the latter characterizing the preponderant number of testicular germ cell tumors. The exact role of the i(12p) isochromosome in testicular germ cell tumor pathogenesis remains to be determined, as is true of the genes involved in or affected by these tumors. Based on presently available information, a hypothetical pathogenetic and oncogenetic model for the development of testicular germ cell tumors is presented.

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APPENDIX D

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Developmental Biology

Production of medakafish chimeras from a stable embryonic stem cell line

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ABSTRACT

▲ Top
▪ Abstract
▼ Introduction
▼ Materials & Methods
▼ Results
▼ Discussion
▼ References

Embryonic stem (ES) cell lines provide a unique tool for introducing targeted or random genetic alterations through gene replacement, insertional mutagenesis, and gene addition because they offer the possibility for *in vitro* selection for the desired, but extremely rare, recombinant genotypes. So far only mouse blastocyst embryos are known to have the competence to give rise to such ES cell lines. We recently have established a stable cell line (Mes1) from blastulae of the medakafish (*Oryzias latipes*) that shows all characteristics of mouse ES cells *in vitro*. Here, we demonstrate that Mes1 cells also have the competence for chimera formation; 90% of host blastulae transplanted with Mes1 cells developed into chimeric fry. This high frequency was not compromised by cryostorage or DNA transfection of the donor cells. The Mes1 cells contributed to numerous organs derived from all three germ layers and differentiated into various types of functional cells, most readily observable in pigmented chimeras. These features suggest the possibility that Mes1 cells may be a fish equivalent of mouse ES cells and that medaka can be used as another system for the application of the ES cell technology.

INTRODUCTION

▲ Top
▲ Abstract
▪ Introduction
▼ Materials & Methods
▼ Results
▼ Discussion
▼ References

Embryonic stem (ES) cell lines directly derived from early embryos (1, 2) offer an *in vitro* system to study the molecular mechanism underlying the retention of pluripotency of cells and to elucidate the mechanisms of cell commitment, determination, and differentiation during embryogenesis. More importantly, they represent an invaluable tool to identify and isolate novel developmental genes by gene trapping and insertional mutagenesis and to study the functions of known genes *in vivo* by gene targeting (3-5). The availability of pluripotent ES cells and the ability to produce chimeras from these cells represent the key steps that link genetic manipulations *in vitro* and phenotypic analysis *in vivo*.

Stable ES cell lines so far have been limited to the mouse (1, 2), despite numerous attempts in other

mammalian (6-11) and nonmammalian species (12). In all of these cases, cultivation of early embryonic cells was possible only for a limited period (7, 12) or their pluripotency only could be maintained partially after extended culture (8, 11). This difficulty has raised concerns that derivation of stable ES lines will remain a unique feature of small rodents and that the ES/knockout technology will be restricted to the mouse.

Small aquarium fish, like the zebrafish and medaka, have attracted considerable attention as a complementary model system for the analysis of vertebrate development (13-15). Their embryos are transparent and easy to observe and manipulate. This accessibility allows the phenotypic analysis of a particular genetic alteration from the earliest developmental stages onward. Therefore, despite the discouraging situation in many other species, attempts have been made toward the derivation of fish ES cell lines. Recently, we and others have established several stable cell lines from medaka blastula embryos (16, 17). In particular, one of these lines, Mes1, has been shown to retain its normal karyotype and pluripotency *in vitro* (18).

Chimeric fish have been generated by blastula transplantation of noncultured embryonic cells in zebrafish (19), trout (20), and medaka (21). However, no chimeras so far have been produced from long term cultured fish cells. Although the molecular mechanisms underlying the body plan and pattern formation are highly conserved among vertebrates, there are some important morphological and physiological differences in early embryonic development between fish and mammals. One major difference is that teleost fish embryos lack zygotic transcription before the midblastula stage, whereas in mice, zygotic expression starts as early as at the two-cell stage. Fish ES cells in culture, on the other hand, are transcriptionally active. For chimera formation, they are introduced into a transcriptionally inert embryonic environment, in contrast to the mouse situation, in which the transcriptional status in ES cells and the host embryo is comparable. Second, fish blastula cells undergo dramatic changes in shape and size upon *in vitro* cultivation, resulting in a 30-fold size difference between host and donor cells. Most importantly, blastula cells in the embryo undergo divisions every 30 min, whereas the doubling time of, e.g., Mes1 cells is 48 h (18). Therefore, the ability to produce chimeras from fish cell cultures remained to be determined. Here, we report the efficient production of viable chimeras from Mes1 cells cultivated for more than 60 passages by cell transplantation into blastula recipients. Using genetic labeling, we show that Mes1 cells differentiate into various types of functional cells and contribute during chimeric embryogenesis to numerous organs derived from all three germ layers.

MATERIALS AND METHODS

▲ Top
▲ Abstract
▲ Introduction
▪ Materials & Methods
▼ Results
▼ Discussion
▼ References

Cell Culture and Transfection. Mes cell lines were derived from blastula-stage embryos of medaka strain HB32C and were maintained under feeder-free culture conditions in ESM3 medium (17, 18). One of these, Mes1, was reinitiated at passages 20-60 from frozen stocks and used for transplantation. Before transplantation, the cells were passaged at least twice in ESM4 medium. The ESM4 medium was modified from the ESM3 medium. ESM4 medium contains 1 mM phenylthiourea (Sigma), higher concentrations of medaka embryo extract (1 embryo/ml), and human basic fibroblast growth factor (10 ng/ml; Tebu, Frankfurt) and lacks human leukemia inhibitory factor.

pCMVgfp is a construct expressing the cDNA for the human codon-optimized, red-shifted mutant green fluorescent protein (hGFP-S65T) from the human cytomegalovirus early enhancer/promoter (CMV). It was derived by removal of a 2-kb *Bam*HI-*Bam*HI fragment containing the neomycin resistance gene from pRc/CMV/GFP, which was constructed by cloning the hGFP-S65T sequence as a *Hin*dIII-*Xba*I fragment from phGFP-S65T (CLONTECH) between the *Hin*dIII and *Xba*I sites in pRc/CMV

(Invitrogen). For genetic labeling, Mes1 donor cells were transfected (22) with pCMVgfp and, 2 days later, checked for transfection efficiency by flow cytometry and used for transplantation.

Cell Transplantation. Single cells were obtained by trypsinization, rinsed, and resuspended in cell transplantation medium (TM: 100 mM NaCl/5 mM KCl/5 mM Hepes, pH 7.1) for microinjection within 2 h at room temperature. Outbred albino medaka strains (i1 and i3) (23, 24) were used as the host. Embryos were collected shortly after fertilization and dechorionated as described (17, 21). For transplantation, they were arranged in a single row on V-shaped 1.5% agarose ramps in Ringer's solution (25) in 6-cm dishes covered with balanced salt saline (21) containing 1% polyethylene glycol. Microinjection of cells into blastulae was performed by using a self-built cell transplantator system mounted on a Leitz micromanipulator. Transplantation needles were made from 1-mm borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, England) with a vertical pipette puller (Bachofer, Reutlingen, Germany). A fine forceps was used to clip the tips of the needles to an opening of 20-30 μm in diameter. The opening was beveled on a capillary sharpener (Bachofer). The needle was filled with TM and connected to the transplantator containing light mineral oil (Sigma). The cell suspension was pipetted onto a flat surface and sucked into the needle. Between 50 and 100 cells were injected into the deep cell layer of each midblastula recipient. Single blastomeres were dispersed (17) and transplanted as described for Mes1 cells, except that the injection needle had a larger opening of 50-60 μm in diameter. The injected embryos were incubated in balanced salt solution-polyethylene glycol mixture at 18°C for the first 2 days and then at 26°C until hatching at day 10. Dechorionated, noninjected control embryos from wild-type and albino strains were reared under the same conditions.

DNA Isolation and PCR. DNA was isolated from single embryos (25) or adult fish (26). PCR primers TyrA (5'-AAGGAGTGCTGTCCAGTGTGG) and TyrC (5'-TGTGCCTGTGGTGATGACGTA) correspond to positions 421 → 441 and 769 ← 789, respectively, of the medaka tyrosinase cDNA (27); TyrB (5'-GGGGGAGTAATTCAGGGTAGA) corresponds to the very 3' terminal sequence of the insert interrupting exon 1 of the tyrosinase gene (28). PCR was run for 35 cycles (94°C for 30 s; 58°C for 30 s, and 72°C for 1 min) in a volume of 25 μl containing 50 ng of DNA and 5 pM of each of TyrA and TyrC (set 1) or TyrB and TyrC (set 2). Ten microliters of PCR products from each set was mixed and separated on agarose gels. The intensity of PCR bands was determined by densitometry on an Enhanced Analysis System (Herolab).

RESULTS

▲ Top
▲ Abstract
▲ Introduction
▲ Materials & Methods
▪ Results
▼ Discussion
▼ References

The Mes1 line was established from the wild-type pigmented HB32C strain. To investigate whether pigmentation is a useful marker to monitor chimera production in the particular donor and host strain combinations available for this study, albino recipients were transplanted with 10-50 blastula-derived, noncultured cells of the donor strain. Seventy percent of the host embryos developed to chimeras showing variegated black pigmentation. Similar results were obtained with blastula-derived, short term cultured cells (3-9 days) (data not shown).

Eight transplantation experiments were performed with Mes1 cells from various passages (27-66). In each of these experiments, pigmented chimeras were obtained. Altogether, 551 embryos were injected, and 263 survived through the pigmentation stage. Fifteen embryos developed one to many wild-type pigment cells. The overall frequency for pigmented chimeras was 6% (Table 1). The melanocytes in these chimeras were found on the head (four cases), inside the head (two cases), on the trunk (two cases), in the eye (two cases), and on the yolk sac (five cases) (Fig. 1). Continuous examination of these chimeras at various developmental stages revealed that these Mes1-derived cells underwent active proliferation and differentiation *in vivo* (Fig. 1 *C* and *D*).

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Table 1. Production of pigmented chimeras from MES1 cells

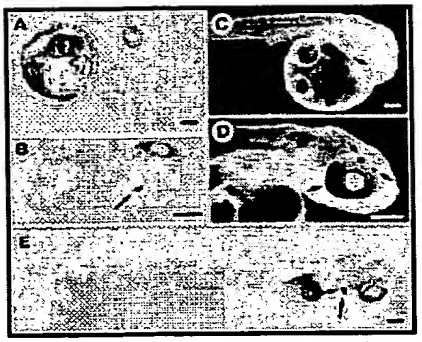

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Fig. 1. Pigmented chimeras obtained from transplanted Mes1 cells. (*A*) Embryos (at day 7) of the donor (*Left*) and host (*Right*) strains. The donor, but not host, strain, shows dark pigmentation in the eye, head, trunk, and yolk sac. (*B–E*) Pigmented chimeras from Mes1 cells transplanted at different passages. Transplantation of cells at passage 31 (234 days of culture) (*B*), at passage 40 (320 days) (*C* and *D*), and at passage 36 (252 days) (*E*). Arrows indicate Mes1 cell-derived wild-type melanocytes in the chimeras. (*B*) A chimera showing a melanocyte on the head. (*C* and *D*) A chimera at different developmental stages. At day 4 (*C*), only a single small pigmented area is evident in the retina. By day 10 (*D*), expansion of this pigmented area to approximately one-third of the whole retina is paralleled by the appearance of two other pigmented areas, indicating proliferation and differentiation of Mes1 cells. (*E*) Pigmented chimeric fry showing melanocytes inside the head in the opercular region. (Bars = 200 μm.)

The melanin-containing pigment cells are the end product of a single one of many cell lineages and thus comprise only a minority of the cells in a developing embryo. Therefore, monitoring pigmentation could lead to an underestimation of chimera frequency. To determine more precisely the chimera frequency and degree of chimerism, an assay was devised to identify donor cells in the host embryos by PCR. The albino strain i1 is homozygous for a deficient tyrosinase gene that carries an 1.9-kb insert within its first exon (28). Accordingly, three PCR primers were designed: TyrA and TyrC define a 369-bp fragment specific for the wild-type donor strain, and TyrB and TyrC generate a fragment of 257 bp unique to the albino strain (Fig. 2*A*). With this assay, it was possible to detect at least 1% contribution of donor-derived DNA in the albino background as determined by serial dilutions of donor in host DNA (Fig. 2*B*). Of hatchlings and fry, 18 of 20 (8-10 days after transplantation) randomly sampled from the third experiment (Table 1) showed the donor-specific band (Fig. 2*C*). The contribution of Mes1-derived DNA per chimera was estimated to range roughly from 2 to 10% (Fig. 2*D*).

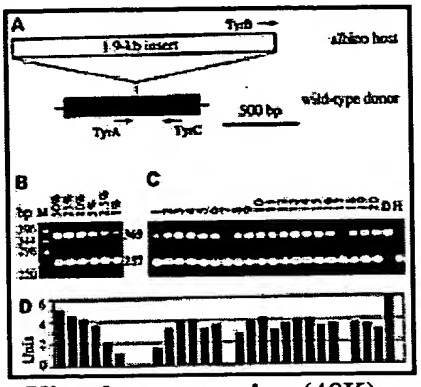

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Fig. 2. PCR detection of Mes1-derived chimeras. (*A*) Schematic structure of the tyrosinase gene in the wild-type Mes1 donor (HB32C) and albino host strains (i1). Only the first exon (black box) of the gene and the 1.9-kb insert (open box) interrupting the exon are shown. PCR primers are represented by arrows. TyrA and TyrC define a fragment of 369 bp specific to the donor strain, whereas TyrB and TyrC give rise to a 257-bp fragment unique to the host strain. (*B*) Sensitivity of the PCR assay. Lane M, 1-kb marker (GIBCO), with sizes shown in base pairs. Numbers in percentages indicate proportions of donor strain-derived DNA diluted with that of the host strain. The 369-bp, donor-specific band is detectable if the donor DNA represents at least 1% of the input DNA. (*C*) Screening of Mes1-injected embryos from the third transplantation experiment (Table 1). The 369-bp, donor-specific band is evident in 18 of 20 hatchlings and fry from Mes1-transplanted embryos (lanes 1-20) and in the donor (lane D) but not the host (lane H) strain. All host embryos display the 257-bp band. Lanes 1-20, pigmented (lanes 1 and 2) and nonpigmented (lanes 3-20) fry (lanes 1-11) and hatchlings (lanes 12-20). The second pigmented chimeric fry (lane 2) is shown in Fig. 1*E*. (*D*) Graphs of intensities of the 369-bp, donor-specific band in the samples shown in *B* and *C*. The intensity of the 369-bp band in the 1% lane (*B*) is equivalent to 1 arbitrary unit.

To address whether Mes1 cells are able to contribute also to other cell lineages in addition to the pigment cell lineage, we derived Mes1 cell transfectants transiently expressing GFP from the CMV promoter. This promoter is strong and shows no tissue-specific restriction of reporter expression in medaka (25). After transfection using a modified calcium phosphate precipitation procedure (22), 7% of Mes1 cells expressed GFP before transplantation as determined by flow cytometry. GFP-transfected cultures were used for injection into blastulae and resultant embryos were scored for GFP-expressing cells by fluorescent microscopy. Of hatchlings and fry, 74 of 78 (10 days after transplantation) that developed from 86 injected embryos were GFP-positive. Thus, injection of only 3-7 GFP-positive cells (7% of 50-100 cells) was sufficient to give a 95% colonization rate. The number of GFP-expressing cells varied from 1 to >50 per chimera (Fig. 3*A*). Such cells were found in 1-5 different compartments (Fig. 3*B*). They were distributed into a wide variety of tissues and organs including the embryonic integument, internal organs, and extraembryonic structures (e.g., yolk sac) (Fig. 3*C*). Similar results also were obtained in another series of experiments, where a LacZ expression construct was used for transfecting Mes1 cells.

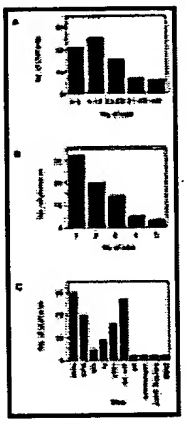

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Fig. 3. GFP-expressing Mes1 cells in 10-day-old chimeras. (*A*) Number of GFP-expressing Mes1 cells per chimera. (*B*) Number of distribution sites of GFP-expressing Mes1 cells per chimera. (*C*) Sites of GFP-expressing Mes1 cells in chimeras. "Head" includes the brain, gill operculum, jaws, otic vesicle; "trunk" includes the tail, spinal cord, notocord, and somites; "eye" means the retina and lens; and "heart" includes the atrium, ventricle, ventral body wall, aorta, and conus arteriosus.

The size and morphology of GFP-expressing cells varied considerably depending on the tissues or organs. Evidence that Mes1 cells developed to terminally differentiated cells from not only the pigment cell lineage but also from other cell lineages came from chimeras having GFP-positive cells in the fin and heart. In the fin, individual large, flat epithelial cells could be identified, and GFP-positive donor cells were indistinguishable from recipient cells in morphology (Fig. 4 *C* and *D*). In the heart, the GFP-expressing donor cells were found in the atrium, ventricle, and associated structures (Fig. 4 *G*, *H*, and *I*). These elongated cells contracted rhythmically. They are assumed to represent differentiated heart muscle cells.

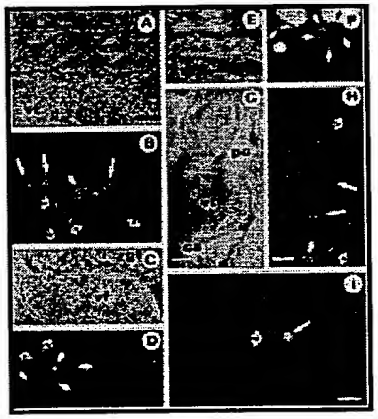

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Fig. 4. Chimeric fry from Mes1 cells transiently expressing GFP. (*A*, *C*, *E*, and *G*) Bright-field micrographs. (*B*, *D*, *F*, *H*, and *I*) Dark-field fluorescent micrographs. (*A* and *B*) GFP-expressing Mes1 cells in the myotome (mt; large arrows) and the ventral fin (vt) as clustered (small arrow) and single (hollow arrows) cells. (*C* and *D*) GFP-expressing Mes1 cells in the ventral fin (vf; arrows) and the gut (gt; hollow arrows). Note the distinct epithelial phenotype of GFP-expressing cells that are morphologically indistinguishable from surrounding recipient cells. The gut is recognizable by its background autofluorescence. (*E* and *F*) GFP-expressing Mes1 cells in the trunk (arrows). GFP-positive cells show green fluorescence and can be distinguished from large, yellow autofluorescent recipient pigment cells. (*G* and *H*) GFP-expressing Mes1 cells in the ventral body wall surrounding the pericard (pc; hollow arrows), the ventricle (vt; large arrows), and conus arteriosus (ca; small arrow) of the heart. (*I*) GFP-expressing Mes1 cells in the atrium (solid arrow) and ventricle (hollow arrow) of the heart. The anterior is to the right, the dorsal side is up. [Bar = 50 μm (*A*-*F* and *I*) and 10 μm (*G* and *H*).]

DISCUSSION

▲ T p
▲ Abstract
▲ Introducti n
▲ Materials & Meth ds
▲ Results
• Discussion
▼ References

This study demonstrates that, in fish, despite a number of dramatic physiological differences between long term cultured blastula-derived cells and developing embryos, it is possible to generate viable chimeras at a high frequency by using the Mes1 cell line in medaka. The present findings also reveal two prominent properties of Mes1 cells. First, these cells retain their *in vivo* pluripotency because they are able to survive, proliferate, and contribute to many different cell lineages and differentiate into functional cell types (e.g., pigment cells, cardiac muscle cells, and fin epithelial cells) during chimeric embryogenesis. Second, they maintain this pluripotency after cryostorage and genetic manipulations *in vitro* because thereafter their ability to participate in host embryogenesis was not restricted. Thus, Mes1 cells appear to be a fish equivalent of murine ES cells (1, 2).

To date, in vertebrates other than the mouse, pigmented chimeras from cultured cells have been reported in chicken (12) and zebrafish (29). In chicken, however, these cells had been cultured only for few passages before transplantation into recipients. In zebrafish, pigmented embryos have been obtained from blastula-derived cells that had been cultured for only 2 days before being used for transplantation. In the latter case, those cell cultures consisted of a significant portion of well differentiated melanocytes. Therefore, it is not clear whether the melanocytes appearing in the albino host embryos resulted from *in vitro*-differentiated melanocytes or from their committed precursors already present in the cell culture injected. In our experiments, Mes1 cells during long term cultivation under the conditions described did not develop a single melanocyte. Thus, the pigmented fry from transplanted albino blastulae are definite chimeras, with their pigmented cells being the descendants of *in vivo* differentiated derivatives of pluripotent Mes1 cells.

In the present study, chimerism was analyzed by three lines of evidence. Black pigment cells of the ES cell donor strain in albino recipients demonstrated the functional contribution of donor cells to the pigment lineage. Melanin pigmentation is therefore a useful marker for the documentation of single Mes1 cells in that specific lineage in living chimeras. This method revealed a chimera frequency of 6%. Because the pigment cell lineage represents only a minor fraction of embryonic tissues, the chimera frequency judged by pigmentation is expected to be an underestimation. Indeed, a significantly higher chimera frequency was obtained by PCR. This method is sensitive enough to detect chimeras harboring as low as 1% of donor cells in any tissue. It revealed a chimera frequency of 90%. Furthermore, it allows for a quantitative estimation of the proportional contribution from donor cells to chimeras. Genetic labeling of donor cells by transfection with a GFP expression construct detected a chimera frequency of up to 95%. It proved the most powerful approach to analyze differentiation of Mes1 cells *in vivo*. Living embryos can be examined continuously, and even a single GFP-expressing donor cell provides a signal sufficient for microscopic observation. Using this approach, we were able to show proliferation and differentiation of transplanted Mes1 cells and their contribution to all major organs in the chimeras.

During transplantation, 50-100 Mes1 donor cells were injected into each recipient embryo. At the time of injection, 7% of Mes1 donor cells were GFP-positive. If injected Mes1 cells were not proliferative, then chimeras would be expected to have only 3-7 GFP-positive cells. That some of the chimeras contained >50 GFP-positive cells indicates a eightfold increase in cell number and thus the active proliferation of Mes1 cells during embryogenesis. Indeed, Mes1 cell proliferation was clearly visible in those chimeras in which the pigmented area expanded continuously.

In mice, the successful production of chimeras is depending not only on the pluripotency of ES cells but also on the genetic compatibility between the ES cell and host strains (30). The availability of numerous different medaka strains will make it possible to determine whether this is also true in fish and to identify the most suitable combinations of donor-host strains. In fish, there are some important morphological

and physiological differences between ES cells and host embryos. Although such differences, as shown here, do not prevent the generation of chimeric embryos, they may influence the frequency of chimeras and the degree of chimerism. Thus, it can be anticipated that optimization of transplantation conditions after testing a plethora of parameters (e.g., adjusting host and donor cell cycles and testing donor-host strains) will further improve the efficacy of chimera production and the contribution of fish ES cells to every cell lineage including the germ line.

The ability of Mes1 cells to differentiate into various functional cell types and their wide distribution into all major organ systems derived from all three germ layers suggest that they are not restricted in their potential to contribute to particular cell lineages. As the first long term cultured ES cell line established from a nonmurine vertebrate species that gives rise to somatic chimeras with high efficacy, the Mes1 line provides a system to study many aspects of cell differentiation *in vitro* and in chimeric embryos in fish as a lower vertebrate system. Its pluripotency and normal karyotype make this cell line a potent source for the production of genetically manipulated cell culture-derived animals by cell transplantation or nuclear transplantation.

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FOOTNOTES

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ABBREVIATIONS

ES, embryonic stem; GFP, green fluorescent protein; Mes, medaka ES; ESM, ES cell medium; CMV, cytomegalovirus.

REFERENCES

▲ Top
▲ Abstract
▲ Introduction
▲ Materials & Methods
▲ Results
▲ Discussion
• References

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